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Evaluation of a plasmid delivery system for production of GnRH and GHRH in the horse and goat

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EVALUATION OF A PLASMID DELIVERY SYSTEM FOR PRODUCTION OF GNRH AND GHRH IN THE HORSE AND GOAT

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TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

The efficacy of a novel plasmid delivery system was assessed for long-term expression of gonadotropin releasing hormone (GnRH) and growth hormone releasing hormone (GHRH) in horses and goats. The efficacy of the technology was demonstrated using 3 novel plasmids: pSEAP [expressing secreted embryonic alkaline phosphatase (SEAP)], pGHRH (expressing GHRH), and pGnRH (expressing GnRH). Geldings were electroporated with a reporter plasmid expressing SEAP in 3 muscle sites. Expression of SEAP, measured from jugular plasma samples, indicated muscle specificity for uptake and expression of the plasmid. Concentrations of SEAP were greatest ($P < 0.05$) after pectoralis injection, which was chosen as the site for electroporation in subsequent studies. In a second experiment, stallions were electroporated with pGHRH or pSEAP to evaluate the effect of long-term GHRH treatment on the growth hormone (GH) axis and testicular function. Stallions treated with pGHRH had increased ($P < 0.05$) plasma concentrations of IGF-I, increased ($P < 0.05$) volume of accessory sex gland fluid, and increased ($P < 0.05$) number of normal spermatozoa in the ejaculate relative to controls. In the third experiment, stallions were electroporated with pGnRH or pSEAP to test the effects of GnRH on the reproductive axis. Treatment with pGnRH increased ($P < 0.05$) plasma testosterone concentrations to d 56 and increased ($P < 0.01$) the LH response to GnRH on d 21, but did not alter ($P > 0.1$) seminal characteristics evaluated after 36 d of treatment. In a final experiment, goat does were treated with pGnRH or pSEAP to assess the effects of GnRH treatment on the reproductive axis during seasonal anestrus. Plasma concentrations of LH and FSH were not affected ($P > 0.1$) by treatment through d 56. Plasma progesterone measurements indicated that ovulation did not occur in does treated with pGnRH or pSEAP. Does treated with pSEAP had increased ($P < 0.05$) plasma SEAP concentrations. In conclusion, electroporatic

plasmid delivery of peptide hormones may serve as an effective technique for expression of protein hormones in the horse and goat.

INTRODUCTION

Traditionally, hormone therapy with protein or peptide hormones, agonists, and antagonists that are short-lived in vivo required frequent bolus injections or depot delivery to elicit long-term effects on physiologic systems. Recently, the ability to transfect DNA into an adult mammal has overcome the barriers of impracticality and economic infeasibility associated with protein hormone therapy. Mammalian plasmid delivery has evolved over the past decade into a safe approach for delivery of DNA and the gene product in vivo. This technique is currently being optimized for application as a minimally invasive intramuscular plasmid delivery system for mammals (Draghia-Alki and Smith, 2003). Electroporatic delivery of plasmids with promoters optimized for mammalian muscle tissue has resulted in successfully high expression levels of protein in mice (Lucas and Heller, 2001; Lesbordes et al., 2002), rats (Terada et al., 2001; Yasui et al., 2001), dogs (Fewell et al., 2001; Draghia-Akli et al., 2003a), and pigs (Draghia-Akli et al., 2003b; Draghia-Akli and Fiorotto, 2004).

In various species, GH treatment has been beneficial on numerous physiologic systems. In the normal horse, GH has been evaluated for its effects on the cardiovascular system (McKeever and Malinowski, 1997), the musculoskeletal and immune system (Malinowski et al., 1997; Smith et al., 1999), and the reproductive axis (Cochran et al., 1999; Aurich et al., 2003; Storer et al., 2005). Growth hormone treatment has resulted in increased granulocyte number and musculation in aged mares (Malinowski et al., 1997), increased number of small follicles on the ovaries (Cochran et al., 1999), and increased accessory sex gland function in stallions (Storer et al., 2005). Researchers have yet to examine the effects of chronic GH treatment on many physiologic systems in the horse.

Treatment with GnRH, the hypothalamic peptide regulating pituitary LH and FSH secretion, and its analogs has shown promise for inducing reproductive recrudescence during

deep anestrus in mares (Johnson, 1986b, 1987; Turner and Irvine, 1991). Problems have arisen with these treatments due to an inability to stimulate physiological GnRH secretion. Native GnRH is short-lived in circulation and requires frequent administration or continuous infusion to mimic physiologic secretion (Johnson, 1986b). Generation of a plasmid expressing GnRH would allow for investigation of the effects of long-term GnRH treatment on various physiological mechanisms including seasonal reproduction.

The following series of experiments was designed to study the potential of plasmid delivery systems as a replacement for repeated or depot-like injections in horses and goats. After an initial experiment to determine the best muscle group for injection and electroporation in horses, two other systems were studied in which potential applications could have positive physiologic and hence commercial impact: 1) GHRH production and secretion for the long-term enhancement of GH secretion in horses, and 2) GnRH production and secretion for the possible enhancement of LH and FSH secretion in seasonally suppressed horses and goats.

CHAPTER I

REVIEW OF LITERATURE

Mammalian Plasmid Delivery Systems

Plasmid delivery is becoming an alternative therapy to routine peptide hormone injections. Traditionally, hormone therapy has been limited to administration of hormones, agonists, and antagonists that are short-lived in vivo and require frequent bolus or depot delivery to elicit desired, long-term effects on physiologic systems. The ability to transfect DNA into an adult mammal has overcome the barriers of impracticality associated with protein hormone therapy. Plasmid gene transfer is ideal when prolonged low-level expression is desirable. In many instances, protein hormone therapy can be inefficient and labor intensive. This is due in part to the cost, availability, and pharmacokinetics of many protein preparations. Combining new plasmid delivery technologies with the elucidation of genetic information for domestic mammals could expand the use of this technique in future therapies. Many factors relating to the efficacy of electroporatic delivery must be determined for maximum utilization of the technology. Particularly, selection of species-specific delivery sites, determination of promoters optimized for specific tissues, and production of functional genetic inserts must be established.

Plasmids are small circular molecules of double stranded DNA attained from bacterial cells (Alberts et al., 2002). Plasmids are widely used as cloning vectors due to their ability to replicate in bacteria (Alberts et al., 2002). Modifications can be made to plasmids by restriction nuclease digestion and ligation to accommodate genetic inserts including mammalian genes (Alberts et al., 2002). Once a gene is inserted into the plasmid, the gene becomes stable and reproducible (Wolff et al., 1990; Acsadi et al., 1991). The incorporated DNA can then be

grown-up in bacterial culture, purified, and transfected to mammalian tissue in vitro or in vivo for expression of encoded peptides (Alberts et al., 2002).

Mammalian plasmid delivery is the process of transferring plasmid DNA into mammals for in vivo genetic expression. The process has evolved over the past decade into a safe approach for delivery of DNA in vivo (Draghia-Alki and Smith, 2003). Other approaches to in vivo gene transfer are being studied with limited success. Viral vectors (Baker et al., 2005; Kapturczak et al., 2005) and transposable vectors (Largaespada, 2003) are two alternative means of gene transfer. These techniques are fairly effective, but generally result in poor efficiency, poor tissue selectivity, or immunogenicity (Largaespada, 2003; Baker et al., 2005; Kapturczak et al., 2005). Plasmids are fairly simple to construct, relatively inexpensive, and of low risk when compared to viral vectors and transposable vectors (Frederickson et al., 2003). Plasmids do not become incorporated into the cell's genomic DNA. Consequently, transgene expression from plasmids is dependent on the turnover rate of the transfected cell type (Lechardeur et al., 1999). This results in a restriction of expression to the site of incorporation. Therefore, the expression is confined, which makes plasmid expression both reversible and safe.

Previous short-comings of plasmid gene transfer were low expression levels brought about by diminished cellular incorporation due to cell death and minimal cellular uptake during transfection (Danko and Wolff, 1994; Tsurumi et al., 1996). New technologies have made it possible to increase DNA incorporation into mature mammalian cells. One of the most promising of these technologies is electroporatic plasmid delivery (Draghia-Akli et al., 2002; Draghia-Akli and Fiorotto, 2004; Khan et al., 2005). Electroporation, also described as electropermeabilization or electrokinetic enhancement, is the process of exposing the transfected tissue to a brief electric pulse. This results in a transient permeability of the lipophilic cell membrane to hydrophilic molecules (Smith and Nordstrom, 2000). Electroporation has been

used extensively for gene transfer, DNA vaccination, and drug delivery in vivo and in vitro (Draghia-Alki and Smith, 2003). Electroporation requires application of conductive electrodes to the transfected tissue. Various electrodes have been studied. These include conductive clamps, tweezers, paddles, and needles. The electrode design is dependent on the nature of the target tissue (Gilbert et al., 1997). It is essential to have the electrical current applied as close to the injected DNA solution as possible. For this reason, specific electrodes are preferred for different tissues to facilitate maximal DNA incorporation.

Previously, the use of electroporation was limited due to damage of affected cells and degradation of the plasmid (Fewell et al., 2001). Electric field intensity, length of pulse, and design of electrodes have been factors contributing to the adverse effects of electroporation (Bureau et al., 2000). Innovations on the technology were reported by Draghia-Alki and colleagues (2002). With their technique, minimal injection site pathology was observed when compared to traditional electroporation (Draghia-Akli et al., 2003b). An electrode needle array delivering a constant current has been most effective at minimizing cellular degradation due to heat produced during electroporation. Also, the addition of polymers to the DNA formulation was shown to improve cellular uptake. Polyvinylpyrrolidone (PVP) and poly-L-glutamate (PLG) are believed to aid in mending the cellular membrane subsequent to electroporation (Draghia-Akli et al., 2002).

Some variation in plasmid production has been attributed to the site of electroporation due to differences in tissue and cell variation (Draghia-Akli et al., 2003b). Long-lived cells seem to respond best to electroporation due to cellular recovery and longevity of expression. Large cell types have been found to be more resilient to the effects of electroporation (Neumann et al., 1999). Muscle cells have been the focus of electroporatic delivery for these reasons. Maximized plasmid uptake and expression are very often muscle specific and may vary from species to

species (Draghia-Alki et al., 2002; 2003a,b). In pigs transfected into the longissimus dorsi and the semitendinosus muscles, the semitendinosus muscle gave the greatest expression (Draghia-Akli et al., 2003b). It was concluded that differences in cellular uptake of plasmid DNA could be determined by muscle fiber types, vasculature of the affected muscle, and subsequent muscle activity.

Although plasmid expression is limited by the life of the transfected cell, in vivo muscle cell transfection has produced expression up to 100 d in duration. In some instances, effects of transfection have been transferred to the offspring (Khan et al., 2002, 2003a). Khan and colleagues (2003a) reported increased weight gain, feed efficiency, and number of GH staining cells in the pituitaries of piglets farrowed by sows treated with a plasmid expressing GHRH. Considering the episomal nature of muscle-specific plasmid transfection, Khan and colleagues hypothesized that GHRH passing through the placental barrier affected the GHRH/GH/IGF-I axis of the offspring, increasing the number of somatotropes and GH secretion postnatally.

Mutated genes and genes from different species can be expressed through plasmid delivery. Studies conducted with electroporation of plasmids encoding a manipulated porcine GHRH gene have revealed that mutated porcine genes will function in dogs (Draghia-Akli et al., 2003a) and cattle (Brown et al., 2004). These results confirm the efficacy of plasmid delivery for interspecies gene transfer. Plasmid promoters dramatically affect the transcription of an encoded gene and can be optimized to be tissue specific and exceed physiologic promoter expression levels (Li et al., 1999). Electroporatic delivery of plasmids with promoters optimized for mammalian muscle tissue have successfully expressed high levels of protein in mice (Lucas and Heller, 2001; Lesbordes et al., 2002), rats (Terada et al., 2001; Yasui et al., 2001), dogs (Fewell et al., 2001; Draghia-Akli et al., 2003a), and pigs (Draghia-Akli et al., 2003b; Draghia-Akli and Fiorotto, 2004). For instance, the promoter SPc5-12 is a synthetic promoter

developed to produce high levels of protein when delivered into mammalian muscle tissue (Li et al., 1999). The promoter is capable of driving transcription in mice, pigs, and dogs with no indication of bias among species (Draghia-Akli et al., 2002).

Growth Hormone in the Horse

Growth hormone is a 191 amino acid protein secreted by the somatotropes of the pars distalis (Hadley, 2000). It was named for its ability to stimulate growth in short statured individuals. Although GH was first discovered for its growth enhancing characteristics, it has since been found to play a role in numerous physiologic systems. Growth hormone stimulates amino acid anabolism, fatty acid mobilization, and collagen deposition to list a few. Growth hormone also stimulates the release of IGF-I from the liver, expanding its influence on mammalian physiology. The two hormones are usually studied in unison due to their tightly linked participation in the GH axis feed-back loop (Hadley, 2000).

In the horse, GH is produced in two distinct cell types in the pars distalis: GH secreting somatotropes and cells producing both prolactin and growth hormone (Rahmanian et al., 1997). Equine GH secretion, determined by radioimmunoassay of jugular plasma samples, is episodic, with pulses superimposed upon basal secretion (Thompson et al., 1992; Stewart et al., 1993). In the foal, plasma concentrations of GH are relatively low at birth. Shortly thereafter, concentrations of GH rise sharply before descending to basal levels (Stewart et al., 1993).

Another rise in GH concentrations occurs at the onset of puberty (Fortier et al., 2005). In adult horses, plasma GH concentrations are greater in stallions than mares (Thompson et al., 1994).

Treatment with GH has revealed many physiologic roles of the hormone. Foals treated with GH develop increased internal organ weights; organs affected by GH treatment include the adrenal gland, kidney, liver, pancreas, spleen, and heart (Kulinski et al., 2002). This indicates a

role of GH or IGF-I on the development of these internal organs. The effects on general health of this pharmacologically-induced hypertrophy of these organs in the horse are unknown, but aerobic capacity and exercise performance is not improved by short-term GH treatment (McKeever et al., 1998; Gerard et al., 2002). Conversely, body weight was not changed in GH-treated developing foals as has been seen in other developing animals (Capshaw et al., 2001). In addition, GH treatment was not effective at altering the anatomical assessments of height at withers, length of body, widths of chest and rump, heart girth, length of head, front or rear cannon lengths, front or rear cannon circumferences, gaskin circumference, or skin thickness (Capshaw et al., 2001).

Growth hormone is involved in the metabolic processes of the horse, and treatment with exogenous GH induces hyperglycemia, hyperinsulinemia, insulin insensitivity, mobilization of fatty acids and decreased urea-nitrogen concentrations (Smith et al., 1999). Increased muscle anabolism and decreased fat deposition are affected by GH as indicated by increased loin-eye area in foals (Kulinski et al., 2002) and increased musculation in aged mares (Malinowski et al., 1997). Induced protein deficiency increases GH secretion, whereas energy restriction has little effect on GH secretion and decreases IGF-I secretion (Sticker et al., 1995b).

Growth hormone has also been evaluated for its effects on the immune system and reproductive axis. Reproductive effects attributed to GH include increased number of small follicles on the ovaries of the mare (Cochran et al., 1999) and increased testicular (Aurich et al., 1999) and accessory sex gland function (Storer et al., 2005) in the stallion. Growth hormone has also been found to stimulate the immune response as is indicated by increased granulocyte number (Malinowski et al., 1997; Guirnalda et al., 2001).

The exact physiologic mechanisms that regulate equine GH synthesis and secretion are still being elucidated. Two hypothalamic neuropeptides are thought to predominately control

GH secretion: somatostatin negatively influences basal GH plasma concentrations, and GHRH stimulates episodic GH secretion (Hadley, 2000). These two hormones, acting in concert, are allegedly responsible for maintaining GH homeostasis (Hadley, 2000).

In the horse, plasma GH concentrations can be increased by various secretagogues (Sticker et al., 2001), feeding (Christensen et al., 1997; Nadal et al., 1997), and exercise (Thompson et al., 1992, 1994; Sticker et al., 1995a). The possible mechanisms responsible for the GH responses to these stimuli may involve somatostatin, GHRH, or both. Pharmacological doses of GHRH are known to dramatically increase GH secretion. Plasma GH concentrations in response to GHRH decrease with consecutive injections and are variable in amplitude (Thompson et al., 1994). Constant infusion of GHRH has not been evaluated in the horse. In humans, constant infusion of GHRH over 24 h does not affect basal plasma GH concentrations, but pulses are augmented and IGF-I concentrations are increased (Vance et al., 1985). This indicates that constant input of GHRH may work to increase intracellular production of GH rather than increase its secretion. From studies on active immunization against GHRH or somatostatin in sheep, it has been shown that many of the secretagogue-induced increases in GH are regulated more by increases in GHRH than by decreases in somatostatin (Magnan et al., 1995). Similar results have been demonstrated in the pig (Farmer et al., 1991). This similarity within species may indicate that the mode of regulation by GHRH and somatostatin would be similar for the horse. These data also reveal that the variability and the lack of understanding associated with the GH axis is consistent among species.

Other factors have been shown to affect GH secretion. Infusion of aspartic acid, glutamic acid, or n-methyl aspartate increases GH in the horse (Sticker et al., 2001). Thyrotropin releasing hormone administration inhibits pharmacological and exercise-induced GH secretion in the horse (Pruett et al., 2003). The exact mechanisms of action for many of these factors are not

well understood. The current dogma is that these factors directly or indirectly alter the opposition of GHRH and somatostatin, resulting in a pulsatile release of GH.

Effects of Growth Hormone on Equine Reproduction

Growth hormone and IGF-I have been found to affect reproduction in most domestic species. Growth hormone deficiency in humans (Kulin et al., 1981) and cattle (Cohick et al., 1996) is associated with delayed puberty, and treatment of the deficiency can normalize the retarded maturation. This is corroborated by evidence in male mice that indicates prepubertal differentiation of Leydig and Sertoli cells is GH dependent (Hochereau-de Reviers et al., 1987). Growth hormone and IGF-I receptors have been identified in the Leydig and Sertoli cells of rat testes (Smith et al., 1987). Furthermore, GH has been found to increase testosterone secretion and spermatogenesis in humans (Kulin et al., 1981) and mice (Chatelain et al., 1991). The effects of GH on reproduction in these species seem to be directly on the gonads, influencing steroid and gamete production directly or indirectly. The indirect influence of GH might be to increase gonadotropin receptors at the level of the gonad, thereby increasing the efficacy of circulating gonadotropins.

As in other species, GH treatment affects equine reproduction. Stallions treated with GH have increased testosterone secretion in response to human chorionic gonadotropin (Aurich et al., 2003) and increased accessory sex gland function (Storer et al., 2005). Similarly, mares treated with GH develop increased number of small follicles on the ovaries (Cochran et al., 1999). These effects of GH and IGF-I on the equine gonads are similar to those seen in other species. In the horse and other domestic species, blood levels of GH increase at the time of puberty (Mauras et al., 1996; Fortier et al., 2005). This is indicative of an influence of GH or IGF-I on the maturation of the gonads and on gametogenesis. This may explain the presence of GH receptors on the testis (Spiteri-Grech and Nieschlag, 1992).

Based on other relationships between equine and nonequine species, IGF-I may also be involved in spermatogenesis. Specific IGF-I receptors have been found on Sertoli cells and Leydig cells of rats (Moore et al., 1993) and humans (Neuvians et al., 2005). Despite these data, the involvement of GH in equine reproduction remains unclear. Hess and Roser (2005) reported conflicting results that GH, IGF-I, or both did not alter Leydig cell testosterone response to equine LH in vitro compared to GH and IGF-I free media. Also, daily treatment with recombinant equine GH for 21 d did not improve the gonadotropin response to a physiologic dose of GnRH, testosterone response to equine LH injection, or sperm morphology (Storer et al., 2005). Further evaluation of the involvement of GH in equine reproduction is needed to clarify these issues.

Gonadotropin Releasing Hormone in the Horse

There are many challenges in equine reproduction that need further study. These include inducing fertile estrus during the winter, pinpointing ovulation, regulating reproductive behavior, and improving seminal quality. Manipulation of GnRH could help elucidate many of the physiologic mechanisms regulating these factors. Investigators have shown that native GnRH and GnRH analogs are promising treatments for inducing cyclicity during anestrus, inducing ovulation, and altering libido. Appropriate GnRH therapies able to accomplish desirable outcomes in these areas have yet to be established.

As reviewed by Hadley (2000), GnRH is a 10 amino acid neuropeptide produced in the hypothalamus of most mammals. Neurons secreting GnRH are directed to the hypophyseal-portal system feeding the pars tuberalis. The primary function of GnRH is the regulation of gonadotropin secretion from the gonadotropes of the adenohypophysis. Stimulation of the gonadotropes has been determined to differentially regulate LH and FSH

secretions that in turn predominately regulate gonadal steroid synthesis and gamete production. Negative feed-back from gonadal steroids regulates secretion of GnRH and the gonadotropins.

In the horse, GnRH neurons are diffusely distributed throughout the mediobasal hypothalamus (Melrose et al., 1994). The short half-life of GnRH in peripheral blood, combined with the large degree of dilution in the venous drainage, make jugular concentrations of GnRH undetectable with routine radioimmunoassay. Measurements of GnRH secretion have been collected through cannulation of the hypothalamo-hypophyseal portal vessel (Alexander and Irvine, 1987; Irvine and Alexander, 1988) and push-pull perfusion of the medial basal hypothalamus (Sharp and Grubaugh, 1987). These data indicate that secretion of GnRH is pulsatile in nature, and generally pulses in GnRH precede pulses in LH and FSH, although peaks in GnRH secretion are more closely related to surges in LH than in FSH. The effects of GnRH on the pituitary have also been studied through disruption of the communication between the hypothalamus and pituitary (pituitary stalk-sectioned; Porter et al., 1997a). In pituitary stalk-sectioned pony mares, pulsatile GnRH administration is necessary to reestablish basal LH secretion.

Release of GnRH is believed to be regulated by environmental stimuli in seasonal animals. The horse is a seasonal breeder, with greatest reproductive activity during the long days of spring and summer. Hypothalamic GnRH content and GnRH secretion are greatest during the summer. Hart and colleagues (1984) reported that content of GnRH in the hypothalamus of mares varied with season and was lowest during midwinter. Conversely, they found no effect of season on number of receptors for GnRH in the pituitary. In another study, Sharp and Grubaugh (1987) reported differences in GnRH concentrations from perfused hypothalami between seasonally anestrous and cyclic mares. These data indicate that

secretion patterns and intracellular stores of GnRH are regulated by season, while the number of GnRH receptors in the pituitary remains fairly constant through out the year.

Many of the physiologic roles of GnRH have been elucidated by experiments based on active or passive immunization against GnRH. In stallions, immunoneutralization of GnRH resulted in decreased sperm number, decreased gonadotropin production, decreased gonadal steroid production, and decreased libido (Malmgren et al., 2001; Turkstra et al., 2005). Similarly, in the mare, GnRH immunization results in decreased gonadotropin production, decreased steroid production, and decreased follicular growth on the ovaries (Garza et al., 1986; Rabb et al., 1990; Imboden et al., 2006). Prolonged treatment with potent analogs of GnRH has produced similar results (Johnson et al., 2002; Johnson et al., 2003). These data reinforce the concept that GnRH is crucial for equine reproduction.

Problems have arisen in attempting to manipulate reproductive aspects of the horse with GnRH due to an inability to augment physiologic GnRH secretion for extended periods of time. Native GnRH is short-lived in circulation and requires frequent administration or continuous infusion to exert long-term effects on reproduction. Conversely, potent agonists of GnRH overwhelm the hypothalamic-pituitary axis and have been associated with down-regulation of gonadotropins (Johnson et al., 2002, 2003).

Extensive research involving GnRH and its analogs has been conducted in seasonally anestrous mares with the goal of inducing ovulation. During seasonal anestrus, daily administration of GnRH stimulates LH and FSH secretion temporarily but does not increase basal plasma gonadotropin levels or induce significant effects on reproduction (Gentry et al., 2002). In addition, the gonadotropin response to daily injections gradually increases over time, indicating a possible GnRH-induced stimulation of intracellular production but inefficacy at increasing basal secretion of gonadotropins (Gentry et al., 2002). To cause an increase in basal

gonadotropin levels, hourly treatment with GnRH, similar to natural secretion, may be the most effective mode of administration, but may not be necessary. Imitating the natural pulsatility of physiologic GnRH secretion has been the most effective method for inducing ovulation in anestrus and transitional mares resulting in ovulation rates of 50 to 80% (Johnson, 1986a, 1987; Johnson et al., 1988; Turner and Irvine, 1991). Johnson (1986a, 1987) reported that hourly injections of GnRH administered to anestrous mares resulted in increased basal LH secretion and ovulation followed by development of a functional corpus luteum (Johnson, 1986a). This method, however, is inadequate for field use due to the excessive labor input.

Alternative methods of GnRH administration have been studied to facilitate on-farm application. To date, attempts at more physiologic GnRH therapies have involved minipumps, slow-releasing compounds, and analogs. In some studies, these treatments appear to be fairly effective at inducing ovulation in anestrous mares. For instance, treatment with the GnRH analog buserelin was reported to hasten the first ovulation of the year in seasonally anestrous mares (Harrison et al., 1990; Mumford et al., 1994). Similar studies involving slow-releasing subcutaneous implants of less potent GnRH analogs and infusion of GnRH have been marginally effective at inducing ovulation during late anestrus and transition, but of the mares that begin to show significant follicular activity, many return to an anestrous state or fail to ovulate (Allen et al., 1987; Hyland et al., 1987; Harrison et al., 1990; Fitzgerald et al., 1993). These studies indicated that the responses to these alternative methods are variable and inconsistent. In all instances, it is implied that a more physiologic regulation of gonadotropin secretion is dependent on the pulsatile secretion pattern of GnRH, which further establishes that pulsatile administration of GnRH is needed to restore physiologic levels of LH and FSH and subsequent ovulation (Porter et al., 1997a). Pulsatile infusion of GnRH results in a greater increase in plasma gonadotropin concentrations than continuous infusion (Becker and Johnson, 1992). This

criterion for inducing gonadotropin secretion with GnRH makes GnRH therapy difficult to implement.

Currently, potent GnRH agonists, GnRH antagonists, and GnRH vaccines are being studied as treatments for inhibiting reproductive function and behavior in mature horses. Potent agonists of GnRH have been effective for short-term inhibition of gonadotropin secretion (Johnson et al., 2002). However, the horse has proved quite resilient to the long-term down-regulation seen in other species. Due to the short-lived deprivation of gonadotropins, these agonists have little efficacy on altering behavior. Vaccines against GnRH have proven to be adequate for decreasing gonadotropin and steroid production but are prone to injection site necrosis and are variable in efficacy (Thompson, 2000; Stout and Colenbrander, 2004). Antagonists of GnRH have been effective in other species, but studies in horses are limited due to the expense of treatment (Stout and Colenbrander, 2004). The GnRH antagonist Cetrorelix inhibits both LH and FSH secretion in vitro, but the antagonist has limited efficacy in vivo (Evans et al., 2002).

In the stallion, pulsatile administration of GnRH manifests a transient rise in plasma LH and testosterone concentrations that is ineffective at altering testicular size or sperm output, but constant infusion of similar low levels of GnRH is not effective at altering reproduction (Blue et al., 1991). Potent agonists induce an initial sharp increase in gonadotropins followed by a short-lived suppression, similar to the pattern displayed in mares, with no noticeable effects on libido and sperm output (Boyle et al., 1991; Johnson et al., 2003). These data indicate that the stallion is similar to the mare, in that pulsatile administration of native GnRH is most efficient at inducing desirable outcomes on reproduction.

Gonadotropin Releasing Hormone in the Goat Doe

Research regarding caprine reproduction is limited due to the lack of goat production in developed countries. This has detracted from the understanding of caprine reproductive physiology. Many of the physiological roles of GnRH in caprine reproduction have yet to be elucidated. Much of the knowledge concerning caprine reproduction has been derived from research in sheep. This is due to the fact that the goat, like the sheep, is seasonally polyestrous, with most breeding activity occurring during the fall and winter. Current knowledge of caprine reproduction has begun to broaden with increasing interest in research endeavors brought on by an increase in desire for goat products in developed countries. Still, many factors affecting caprine reproduction have yet to be determined. Treatment with GnRH offers opportunities for improving caprine reproduction.

The caprine estrous cycle is generally 21 d in length, with standing estrus displayed in the final 24 to 48 h (Rubianes and Menchaca, 2003). Similar to other farm species, the reproductive cycle consists of 2 main phases. Progesterone predominates during the luteal phase, generally15 d long (de Castro et al., 1999). Elevated levels of FSH during the luteal phase give rise to the development of follicular waves. Goats display a wave-like pattern of follicular growth emerging every 5 to 7 d, resulting in an average of 4 waves over the 21 d cycle (Ginther and Kot, 1994). In the follicular phase, estrogen rises before ovulation in association with the emergence of a dominant, ovulatory follicle. Estrogen exerts a biphasic effect on LH secretion by acting at the level of the hypothalamus and pituitary in a manner similar to that described for the cow. The initial effect of estrogen is to suppress LH secretion before stimulating the final ovulatory surge (Sakurai et al., 1995).

Secretion of GnRH from the hypothalamus is believed to be the primary influence for changes occurring during the estrous cycle. For example, progesterone implantation for an

extended period results in a surge in gonadotropins upon removal. This is blocked by immunization against GnRH and can be restored by pulsatile administration of GnRH (Sakurai et al., 1992). These data indicate a progesterone inhibition of GnRH secretion, and a stimulatory effect of GnRH at the level of the gonadotrope. Furthermore, GnRH antagonists have been found to decrease gonadotropin secretion and follicular growth indicating a direct or indirect role of GnRH at the level of the pituitary and gonad (Lopez-Alonso et al., 2005). These data further substantiate the importance of GnRH in the regulation of gonadotropin secretion and gonad function, as well as demonstrate the similarities in the caprine estrous cycle and that of other farm species.

The increasing knowledge of the physiological role of GnRH in caprine reproduction is proving beneficial for managing reproduction. For instance, GnRH therapy has shown promise for inducing cyclicity during anestrus. Various methods have been studied regarding induction of ovulation in anestrous does. Of these, the most effective involve progesterone pessaries in conjunction with GnRH or gonadotropins (Bretzlaff and Romano, 2001; Whitley and Jackson, 2004). Progesterone-priming in conjunction with hourly injections of GnRH induced an LH surge and ovulation in seasonally anoestrous goats (Knight et al., 1988). In many instances, the estrus was infertile after a labor intensive treatment regimen. In few cases, GnRH analogs have proven successful at inducing ovulation. Buserelin acetate, a relatively less potent analog of GnRH, administered in conjunction with progesterone-priming, has induced LH secretion and ovulation (Medan et al., 2002). Similar to other domestic species, continuous infusion of GnRH results in down-regulation of gonadotropin secretion in goats (Porter et al., 1997b). This limits the use of potent analogs or bolus injections in caprine synchronization schemes. Chorionic gonadotropins have shown promise at inducing ovulation in conjunction with progesterone

treatment, but generally produce antibodies with continued use (Ritar et al., 1984; Bramley et al., 2004). A long-term physiologic GnRH therapy might be applicable in this area.

Rationale for Present Experiments

Significant progress has been made in the discovery and development of alternative hormone therapies. Plasmid-mediated gene transfer is a novel delivery technique developed in recent years to improve hormone therapy. This technology is being applied to increase efficacy or improve practicality of hormone delivery. Unique plasmids can be constructed to accommodate a specific protein at a specific expression level. In the horse industry, GnRH and GHRH have drawn interest for their effects on reproduction and performance. Plasmid-mediated gene transfer technologies could be applied to improve the utilization of these hormones in equine production. For these reasons, the experiments reported herein were designed 1) to test known plasmids and their delivery technologies in the horse and 2) to develop and apply novel plasmids in the horse and goat with the goal of increasing reproductive efficiencies of these two species. To this end, plasmids were constructed to express GHRH and GnRH in muscle tissue. A novel electroporation technique was evaluated for transfection efficacy in the horse and goat. Stallions were treated with plasmids expressing GHRH and GnRH to evaluate the effects on the reproductive axis and seminal characteristics. Subsequently, goat does were treated with the GnRH expressing plasmid to evaluate the effects of GnRH on inducing ovulation during the non-breeding season.

CHAPTER II

REGULATION OF THE EQUINE GROWTH HORMONE AXIS BY ELECTROPORATIC DELIVERY OF A PLASMID ENCODING GHRH: EFFECTS ON SEMINAL CHARACTERISTICS

Introduction

Growth hormone treatment of the horse has been evaluated as an enhancer of the cardiovascular system (McKeever et al., 1997), the musculoskeletal and the immune systems (Malinowski et al., 1997; Smith et al., 1999), and the reproductive axis (Cochran et al., 1999; Storer et al., 2005). Only a few beneficial results have been attributed to GH treatment of horses, which include increased granulocyte number and musculation in aged mares (Malinowski et al., 1997), increased number of small follicles on the ovaries (Cochran et al., 1999) and increased accessory sex gland function (Storer et al., 2005). No one yet has examined the effects of GH on pathologies including stallion subfertility, rhabdomyolysis, and laminitis, due in part to the cost and availability of GH for chronic treatment. Traditional hormone therapies in domestic animals and humans involve routine intramuscular injections of short-lived protein preparations. These treatments are often painful, distressful, and inconvenient for both the treated animals and humans administering the injections. A relatively new technology, based on mammalian plasmid delivery, has made it possible to provide months of therapy with a single injection. A plasmid can be injected into muscle tissue where it functions comparable to chromosomal DNA. Previous shortcomings of the technology were low expression levels brought about by minimal cellular incorporation (Danko and Wolff, 1994; Tsurumi et al., 1996). Newer technologies have made it possible to incorporate DNA into mature mammalian cells for production of encoded peptides *in vivo*. The objective of the present experiment was to evaluate a novel intramuscular plasmid-mediated hormone delivery system for the horse consisting of a

plasmid encoding GHRH, which if effective, would provide information on the effects of a long-term elevation of plasma GHRH concentrations on the GH axis and reproductive axis of the stallion.

Materials and Methods

Experiment 1. Assessment of muscle groups for plasmid delivery. Nine light-horse geldings, 5 to 15 yr of age, weighing between 500 and 600 kg [body condition scores (BCS; Henneke et al., 1983) of 6 to 8], were maintained on native grass pasture with supplemental grass hay as needed to maintain body condition. On d 0 (April 19, 2005), 3 geldings received electroporatic delivery of the plasmid, pSEAP (Figure 2.1; a muscle-specific plasmid expressing secreted embryonic alkaline phosphatase; provided by ADViSYS, Inc. Woodlands, TX) into one of 3 unique muscle groups [splenius (neck), pectoralis (chest), and semitendinosus (rump)].

The plasmid was delivered as 2 mg of DNA in 2 mL WFI (water for injection) + 0.1% PLG (poly-L-glutamate) into the select muscle site. In preparation for injection and electroporation of the plasmid, horses were first sedated with 1.1 mg/kg of xylazine and 0.02 mg/kg of butorphanol administered i.v. to effect; the delivery site was then clipped and sanitized with chlorahexadine. Subsequently, the electrode needle array portion of the Electrokinetic Device (EKD; provided by ADViSYS, Inc. Woodlands, TX) was inserted. The needle electrodes consisted of a mounted circular array (1 cm diameter) of 5 evenly spaced, 21-gauge solid stainless steel electrodes (2 cm in length) mounted on a nonconductive plastic disc. After insertion of the EKD, the plasmid was injected i.m. via a 21-gauge needle into the center of the needle array. Eighty seconds after plasmid injection, a constant current was delivered at 0.4 to 0.6 amps, 3 pulses, 52 msec each, with 1 sec between pulses.

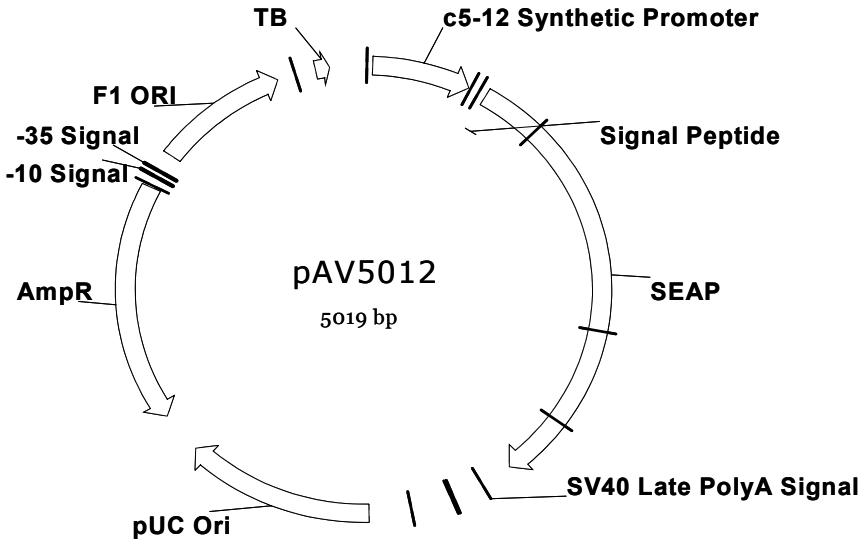

Figure 2.1. Map of secreted embryonic alkaline phosphatase plasmid (pSEAP). The 5019 bp circular plasmid contains the c5-12 synthetic promoter and the gene for SEAP (provided by ADViSYS, Inc. Woodlands, TX). The plasmid is used extensively as a reporter for *in vivo* and *in vitro* studies.

Jugular blood samples were collected via venipuncture on d 0 (before injection), 3, 7, 10, 14, 17, 21, 24, 27, and 30 relative to treatment. Blood samples were immediately centrifuged (1,600 x g at 5°C for 15 min) and plasma was harvested and frozen at -15°C until assay. Plasma from daily samples was analyzed for SEAP by chemiluminescent assay (Phospha-Light System, Applied Biosystems, Bedford, Massachusetts).

Experiment 2. Effects of electroporatic delivery of a plasmid encoding for GHRH. Twelve light-horse stallions, 2 to 24 yr of age, weighing between 500 and 600 kg (BCS of 4 to 6), were paired based on age and weight. Stallions were then randomly allotted so that each treatment was represented within each pair. Each stallion received either i.m. electroporatic delivery of pGHRH (Figure 2.2; a muscle-specific plasmid expressing porcine GHRH; provided by ADViSYS, Inc., Woodlands, TX; n = 6) or pSEAP (n = 6). The plasmids were delivered as 2 mg of DNA in 2 mL WFI + 0.1% PLG as described in Experiment 1.

Blood samples were collected via jugular venipuncture into heparinized tubes on d 0, 1, 5, 8, 12, 15, 20, 22, and 29 relative to injection for assessment of GHRH and SEAP concentrations (June 19, 2006).

On d 22, all stallions received indwelling jugular catheters for frequent blood sampling to characterize the GH secretory patterns of the 2 groups. Catheters were inserted at -60 min, and blood samples were collected into a heparinized tube at 0, 10, 20, 30, 45, 60, 90, 120, 150 min.

Blood samples were immediately centrifuged (1,600 x g at 5°C for 15 min) and plasma was harvested and stored frozen (-15°C) until assay. Plasma samples were analyzed for SEAP by chemiluminescent assay (Phospha-Light System). Concentrations of GH and IGF-I were determined by RIA previously validated for horse tissues as described by Thompson et al. (1992) and Sticker et al. (1995b), respectively.

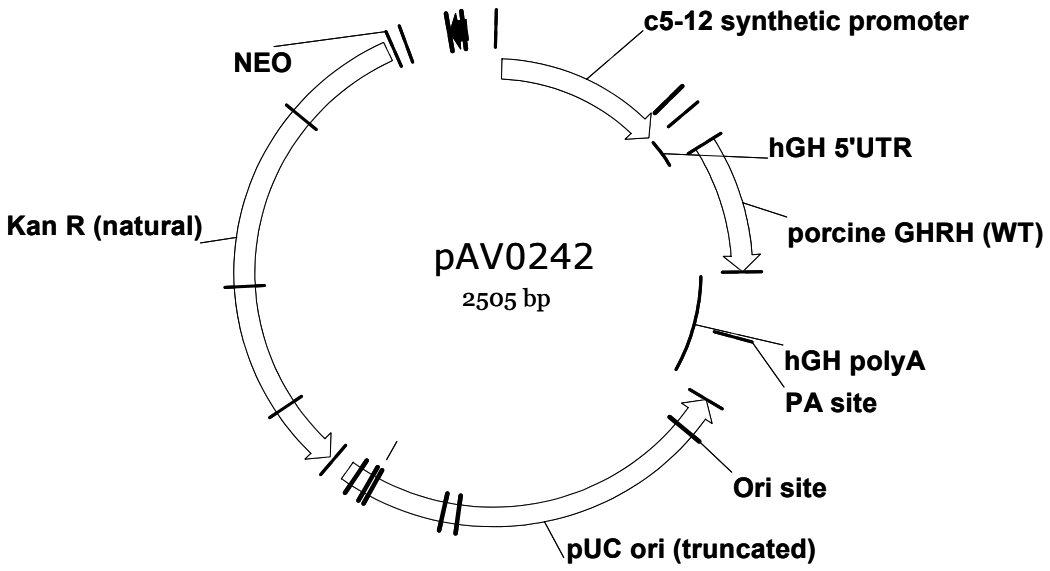

Figure 2.2. Map of growth hormone releasing hormone plasmid (pGHRH). The 2505 bp circular plasmid contains the c5-12 synthetic promoter and the gene for GHRH (provided by ADViSYS, Inc. Woodlands, TX; Draghia-Akli et al., 1999).

Semen was collected from all stallions every other day for 14 d beginning 3 wk before treatment and then again beginning on d 30 relative to treatment. Semen evaluation was conducted on the last 4 ejaculates from each stallion (Pickett et al., 1976). Gel volume, gel-free volume, progressive motility, concentration, and general sperm morphology were assessed for each ejaculate. Morphological characteristics evaluated were head, midpiece, and tail abnormalities and proximal and distal droplets. Gel-free semen was fixed in 2% buffered formol-saline and 100 sperm from each ejaculate were analyzed with phase contrast microscopy (Blanchard et al., 2003).

Data from blood collections were analyzed by ANOVA for effects of treatment and time and the treatment by time interaction as a randomized block design with repeated measures using SAS mixed procedure (SAS Institute Inc., Cary, NC). Seminal characteristics were analyzed for effects of treatment and period and the treatment by period interaction using SAS mixed procedures. Differences at individual time points were determined by the least significant difference (LSD) test (Steel et al., 1980) when a significant F ($P < 0.05$) was detected. Plasma concentrations of SEAP in Experiment 2 were adjusted as percent change from individual pretreatment means to account for individual variation in assay baselines.

Results

Visual appraisals of the electroporation sites were conducted in both experiments. No noticeable swelling was detected beyond 24 h after injection in any of the geldings or stallions.

Experiment 1. Concentrations of SEAP increased ($P < 0.01$) in jugular plasma after electroporation of the SEAP-expressing plasmid, pSEAP, at all 3 muscle sites. The greatest ($P < 0.05$) response was in geldings that received the plasmid injection into the pectoralis muscle (Figure 2.3).

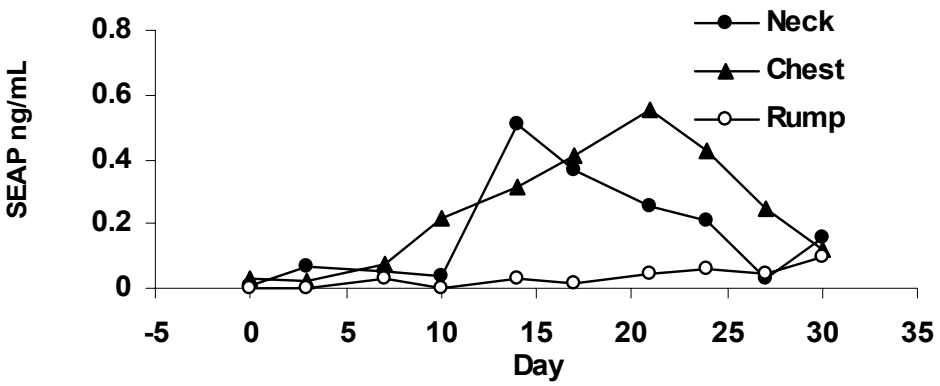

Figure 2.3. Mean plasma concentrations of SEAP for samples collected twice weekly in response to electroporation with pSEAP (time 0) in the neck (splenius), chest (pectoralis), or rump (semitendinosus) muscle. Concentrations of SEAP increased over time ($P < 0.05$) and were greatest ($P < 0.05$) when delivered in the chest. The pooled SEM was 0.11 ng/mL.

Experiment 2. Plasma GH concentrations measured by frequent blood sampling on d 22 indicated that stallions treated with the GHRH-expressing plasmid maintained a constant average GH concentration throughout the blood sampling period, while GH concentrations in stallions treated with the pSEAP plasmid declined ($P < 0.05$; Figure 2.4). However, in plasma harvested twice weekly, GH concentrations were not different between treatment groups (Figure 2.5). Concentrations of IGF-I were increased ($P < 0.05$) but remained within the physiological range in stallions treated with pGHRH (Figure 2.5). As expected, SEAP concentrations were elevated ($P < 0.05$) in stallions treated with pSEAP (Figure 2.5) but not in stallions receiving the GHRH plasmid.

Semen evaluation demonstrated that stallions treated with pGHRH had a decrease ($P < 0.05$) in gel-free volume, an increase ($P < 0.05$) in percentage of normal spermatozoa, a decrease ($P < 0.05$) in percentage of head abnormalities, and a tendency ($P < 0.1$) of increased volume of gel (Figure 2.6). Other semen characteristics (i.e., concentration of spermatozoa, midpiece abnormalities, and tail abnormalities) did not differ between treatment groups.

Discussion

In Experiment 1, plasmid injection i.m. followed by electroporation proved to be an effective delivery system for the pSEAP plasmid in the horse. As indicated by assay of peripheral SEAP concentrations, i.m. electroporation was muscle-specific in the horse, but seemed to be differentially expressed, or differentially absorbed, in the 3 different muscle groups. The response to plasmid electroporation seemed to be fairly reliable and consistent when conducted in the pectoralis muscle. Decreased means for SEAP concentrations in geldings treated in the splenius and semitendinosus indicated a greater variation among individual subjects and a relatively minimal response amongst the group. Efficacy of the treatment and accessibility at the site of the pectoralis make it an ideal treatment site for application of

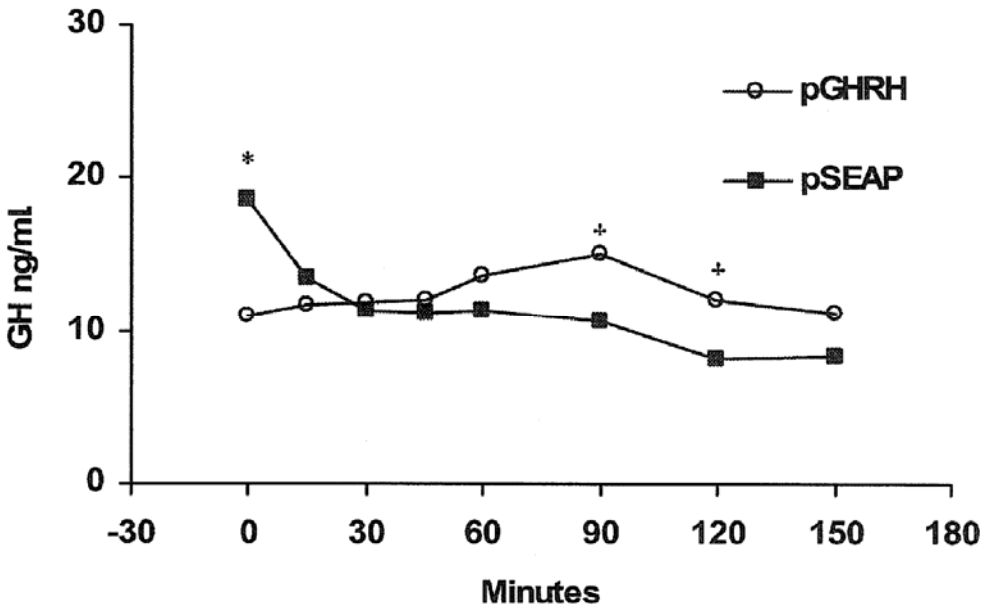

Figure 2.4. Mean plasma concentrations of GH in frequent samples collected on d 22 in pGHRH- and pSEAP-treated stallions. Differences between groups for plasma GH concentrations at individual time points are indicated (*$P < 0.05$; +$P < 0.1$). Pooled SEM was 2.3 ng/mL.

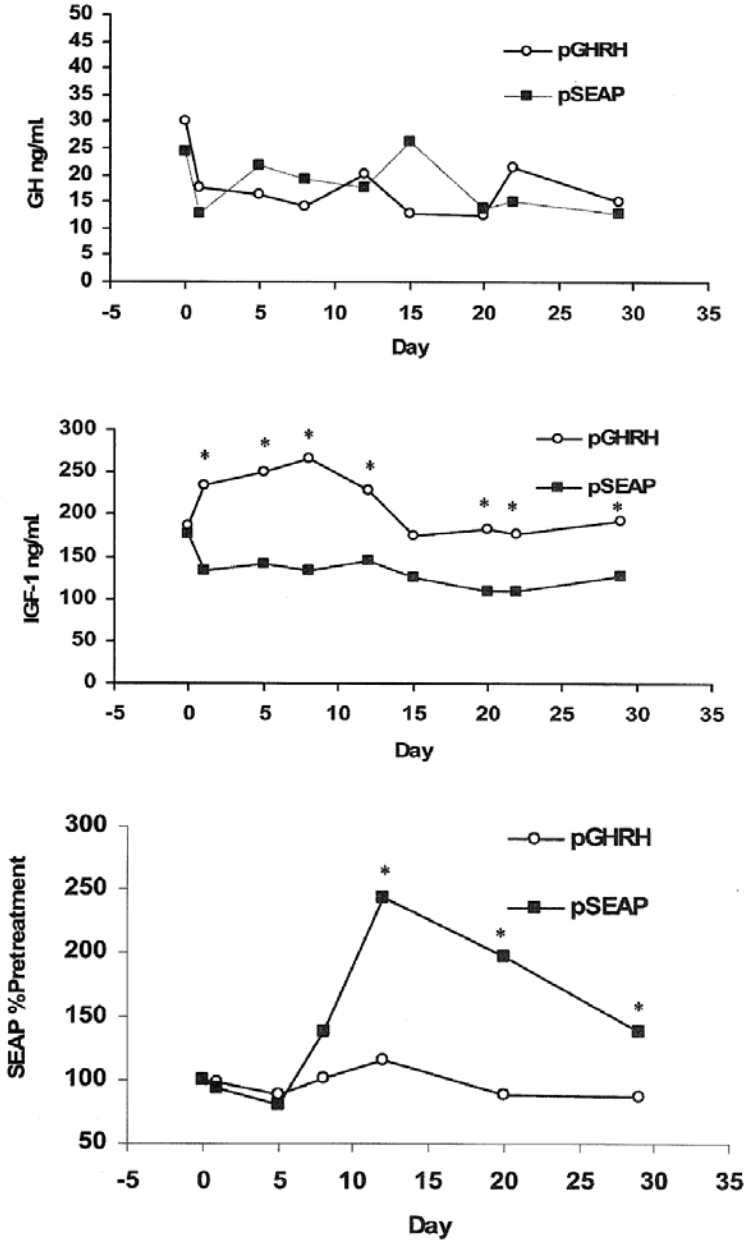

Figure 2.5. Mean plasma concentrations of GH and IGF-I and % of pretreatment SEAP concentrations in samples collected twice weekly in response to electroporation with pGHRH or pSEAP on d 0. Concentrations of GH did not differ between groups. Concentrations of IGF-I were increased ($P < 0.05$) in pGHRH-treated stallions, and concentrations of SEAP increased ($P < 0.05$) in pSEAP-treated stallions. Differences between groups ($P < 0.05$) are indicated by asterisks. Pooled SEM were 5.8, 33, and 27 ng/mL for GH, IGF-I, and SEAP concentrations, respectively.

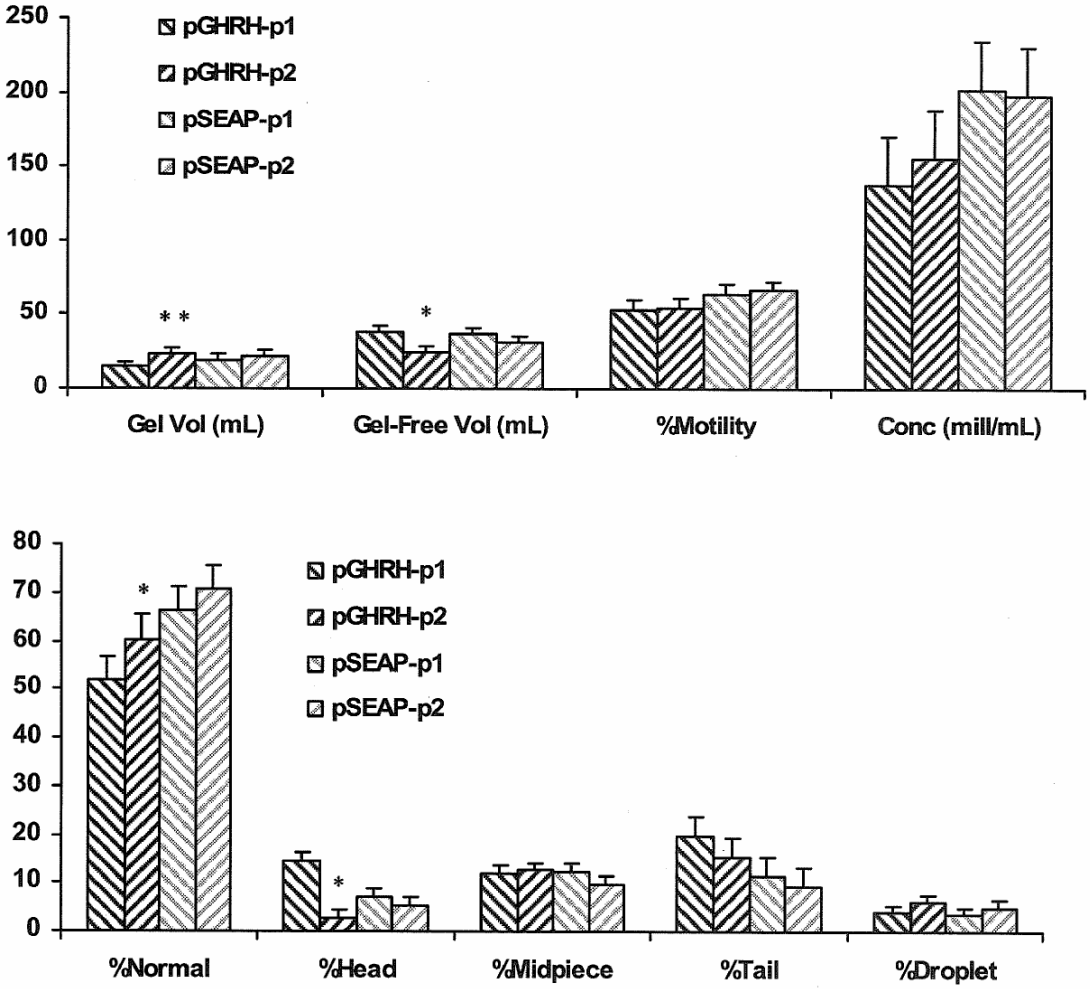

Figure 2.6. Mean seminal characteristics for pGHRH-and pSEAP-treated stallions from ejaculates collected 3 wk prior to treatment (p1) and on d 30 relative to treatment (p2). Stallions treated with pGHRH had a decrease ($P < 0.05$) in gel-free volume, an increase ($P < 0.05$) in percentage of normal spermatozoa, a decrease ($P < 0.05$) in percentage of head abnormalities, and a tendency ($P < 0.1$) of increased volume of gel. Error bars represent pooled SEM and asterisks indicate differences from other groups and periods (* = $P < 0.05$ and ** = $P < 0.1$).

this technique in the horse industry. Thus, the pectoralis was chosen as the site for electroporation in Experiment 2.

It should be noted that SEAP, being foreign to the adult horse, is recognized as such and is eventually cleared by the immune system (Gronevik et al., 2005). This results in a sharp, transient increase in plasma concentrations of SEAP (after electroporation) followed by a similarly sharp reduction. In pigs, electroporation of a plasmid encoding the SEAP gene resulted in peak plasma SEAP concentrations by d 10 before beginning to decline (Khan et al., 2003b; Draghia-Akli and Fiorotto, 2004).

The pSEAP plasmid was chosen in Experiment 1 as a model with known efficacy in other species (Draghia-Akli et al., 2002; Gronevik et al., 2005) that provided an easily measurable protein product not normally found in adult stallions. The pSEAP and pGHRH plasmids contain a similar synthetic muscle-specific promoter and therefore would be expected to be transcribed in a similar fashion. Used as a control in the second experiment to confirm electroporation success, pSEAP expressed SEAP in stallions under the same conditions as pGHRH. This would indicate that pGHRH should have expressed GHRH at a similar rate in the stallions in Experiment 2. Assuming that the expression of the 2 proteins was similar, plasma concentrations of GHRH would be expected to be longer lived than those of SEAP due to its lack of immunogenicity in the horse.

The pulsatility of plasma GH secretion may have been a factor in measuring changes associated with GHRH stimulation directly. Therefore, in Experiment 2, IGF-I concentrations were considered indicative of pGHRH efficacy. The response in IGF-I concentrations is consistent with a prolonged expression of GHRH beyond 30 d. Variation in IGF-I concentrations in the pGHRH-treated stallions was considerably greater than that of the pSEAP group, possibly indicating the need for further development of the delivery technique. Variation

in the increase in IGF-I was likely due to differential expression between individuals or compromised cellular incorporation of the plasmid.

Plasma GH concentrations, evaluated twice weekly, were pulsatile and did not indicate differences associated with pGHRH treatment. This is consistent with studies in humans. Vance and colleagues (1985) reported that GHRH infusion did not affect basal plasma GH concentrations. Conversely, GH pulses were augmented and IGF-I concentrations were increased. In hindsight, the current experiment might have been improved by administration of a GH secretagogue to evaluate any perturbation or augmentation of the GH response in pGHRH-treated stallions. As an alternative, frequent blood sampling was conducted to better characterize the GH response to pGHRH.

Frequent blood sampling on d 22 indicated a differential response over time in plasma GH concentrations. The initial difference in GH concentrations may have been misleading due to the influence of several control stallions exhibiting pulses in GH just prior to the sampling period. It appeared that concentrations of GH were declining, perhaps after abrupt secretion driven by excitement at catheterization (stallions were catheterized 1 hr prior to the first blood sample). Previous studies have shown that epinephrine administration, physical exercise, or restraint with a twitch cause acute increases in plasma GH concentrations (Thompson et al., 1992). Therefore, samples collected later in the sampling period would better represent basal GH secretion for the groups. By 120 min, the variability in the samples had decreased, and basal secretion was greater in the pGHRH-treated group.

Previously, it was reported that stallions treated daily with recombinant equine GH had increased accessory sex gland function (Storer et al., 2005). Stallions in the present study only exhibited a tendency toward increased volume of gel. The decrease in number of head abnormalities and gel-free volume reported herein are in contrast to that previously reported.

Head abnormalities, in the present study, were analyzed using phase contrast imaging which is more sensitive to head abnormalities than the eosin-nigrosin stain used in the earlier study. The improved technique may have detected abnormalities that were overlooked in the previous study. Also, the mean number of pretreatment head abnormalities was greater in the pGHRH-treated group. This was partially due to one stallion that displayed an abnormally high number of sperm head abnormalities. In post treatment morphological assessments, the number of head abnormalities displayed by this stallion was dramatically reduced. In rats, GH treatment improved recovery of spermatogenesis after pharmacologically induced testicular damage (Satoh et al., 2002), and IGF-I treatment improved the morphology of epididymal spermatozoa (Vickars et al., 1999). This may indicate the ability of GH and/or IGF-I to improve viability and longevity of spermatozoa in the epididymus. There are no reports of decreased gel-free volume in response to GH treatment. Further studies are needed to clarify this issue.

In conclusion, it has been shown that electroporatic-enhanced plasmid delivery, shown to be effective in other species (Brown et al., 2004; Draghia-Akli and Fiorotto, 2004), can be used to cause the production of the directed protein (SEAP) in the horse. Of the muscle groups tested, the pectoralis produced the greatest amount of protein measured in the blood. In addition, this novel plasmid-mediated treatment, used to deliver pGHRH DNA into the muscle, resulted in an enhancement of plasma IGF-I concentrations for at least 30 d, and allowed for the evaluation of long-term effects of elevated IGF-I levels on stallion reproduction. Such treatments may be beneficial in the equine industry for improving ejaculates of stallions collected for artificial insemination or natural breeding. Further investigation involving this plasmid needs to be conducted, particularly in the subfertile stallion. This technique should also be useful for evaluation of other hypothalamic and pituitary hormones by incorporating other cDNA into the plasmid for evaluation of the effects on other physiological systems and pathologies in the horse.

CHAPTER III

REGULATION OF THE STALLION REPRODUCTIVE AXIS BY ELECTROPORATIC DELIVERY OF A PLASMID ENCODING GNRH

Introduction

Enhancement of fertility in the normal and subfertile stallion could be economically beneficial to horse breeders. Many popular stallions are limited by their reproductive capacity and cannot meet the demands of the industry. Increasing the libido and sperm output of these stallions could increase the number of breedings attained in a particular breeding season. Furthermore, stallion reproductive capacity is substantially reduced during the winter. Gonadotropin releasing hormone (GnRH) therapy has shown promise as a treatment for improving gonadotropin and testosterone secretion in these scenarios. Chronic, pulsatile administration of GnRH increases LH and testosterone secretion but is less effective on semen motility in stallions (Blue et al., 1991). Pulsatile therapy is, however, impractical due to the labor needed and the long-term nature of the treatment. Long-lasting, potent analogs of GnRH initially increase gonadotropin concentrations but subsequently down-regulate gonadotropin production (Boyle et al., 1991; Johnson et al., 2003). The aim of the current experiment was to evaluate a novel plasmid-mediated delivery system for extrahypothalamic GnRH expression. The plasmid includes a muscle specific promoter followed by a construct comprised of bases 1-99 of the porcine GnRH gene (Weesner et al., 1997). The plasmid was designed for long-term expression of GnRH with the goal of improving the production of gonadotropins and testosterone in the stallion.

Materials and Methods

Plasmid preparation. The plasmid designed for this experiment (pGnRH) was generated from pGHRH, a muscle-specific plasmid expressing porcine GHRH provided by ADViSYS, Inc.

Woodlands, TX (Draghia-Akli et al., 1999). The plasmid was reconstructed to replace the existing GHRH cDNA sequence with the cDNA encoding GnRH by enzymatic cleavage at the 5' Nco I and 3' Hind III sites. A new cDNA insert was constructed for alternative expression of GnRH based on the porcine gene for GnRH (Weesner et al., 1997; GeneBank accession #L32864). The constructed insert included bases 1 through 99 of the porcine GnRH gene modified to include an Nco I restriction site at the 5' end and two 3' stop codons in conjunction with a Hind III restriction site. The complete cDNA insert was generated by PCR from two overlapping primer templates [5'-CCATGGAGCCAATTCCGAAACTTCTAGCCGGACTT CTGCTGCTG**ACTCTGTGTGTAGTGGGCTGC**-3' (65 bp) and 5'-AAGCTTTCATTATCC-AGGGCGCAATCCATAGGACCAGTGTTGGCTGGA**GCAGCCCACTACACACAGAGT**-3' (69 bp)] and extended with primers designed for annealing at the generated 5' and 3' ends [5'-CCATGGAGCCAATTCCGAAA-3' (20 bp) and 5'- AAGCTTTCATTATCCAGGGCG-3' (21 bp)]. The resulting PCR product was cloned using the TOPO XL PCR Cloning Kit (Gibco Invitrogen). Colonies were selected by kanamycin resistance and plasmid DNA was purified via QIAfilter Plasmid Midi Kit (Qiagen Inc., Valencia, CA). The sequence of the purified DNA was then verified by PCR by personnel in the Division of Biotechnology and Molecular Medicine, Louisiana State University School of Veterinary Medicine. Both the TOPO-XL vector and the pGHRH plasmid were simultaneously double-digested with restriction endonucleases Nco I and Hind III (Gibco Invitrogen) for 24 h at 37º C. The digested plasmid and insert products were then separated by agarose gel electrophoresis and purified with the PureLink Gel Extraction Kit (Gibco Invitrogen). The products were then ligated using the DNA Ligation Kit Ver.1 (Takara Mirus Bio Inc., Madison, WI). The resulting plasmid was transfected into a DH alpha E.coli cell line and selected based on kanamycin resistance. Plasmid DNA was purified from resulting colonies with QIAfilter Plasmid Midi Kit (Qiagen) and the sequence verified by PCR. Select

colonies were grown up and plasmid DNA was purified with EndoFree Plasmid Giga Kit (Qiagen).

Transfection of mouse myoblast cells. Mouse myoblast cells (Sol8), obtained from the American Type Culture Collection (Manassas, VA; CRL-2174), were transfected with the plasmid to evaluate the efficacy of the plasmid in muscle tissue. Prior to transfection, cells were grown to 70 to 80% confluency in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 20% fetal bovine serum (Gibco Invitrogen) and 1% penicillin-streptomycin (Gibco Invitrogen) at 37°C in a humidified environment of 5% CO_2 and 95% air. At 80% confluence, media was replaced with Dulbecco's modified Eagle's medium supplemented with 2% heat-inactivated horse serum (Gibco Invitrogen) and 1% penicillin-streptomycin (Gibco Invitrogen). Cells were then transfected with 4 µg of pGnRH (n = 4), or no DNA (n = 1) via Fugene 6 Transfection Reagent (Roche Applied Sciences, Indianapolis, IN). Transfected cells were incubated for 72 h at 37°C in a humidified environment of 5% CO_2 and 95% air. Media was collected at 72 h, and GnRH expression was verified by RIA of culture media for GnRH.

Treatment of stallions. Ten reproductively sound stallions ranging in age from 2 to 25 yr were used. They were allotted to 3 groups such that average age and weight were similar for the groups. On d 0, stallions in the first group received i.m. electroporation of 2 mg pGnRH in 2 mL of vehicle (WFI + 0.1% PLG; n = 3); the second group received 4 mg of pGnRH in 2 mL vehicle (n = 3); and the third group received 2 mg of pSEAP in 2 mL of vehicle (n = 4) as described for Experiment 1. Preparation and anesthesia of the stallions and injection and electroporation of the plasmids were performed as described in Chapter II.

Blood samples were collected from all stallions twice weekly beginning 1 wk before treatment through 6 wk after treatment. On d 21, stallions were fitted with an indwelling jugular

catheter. One hour later, all stallions received a challenge injection of GnRH (0.1 μg/kg of BW, i.v.; Sigma), and blood samples were collected at -20, -10, 0, 10, 20, 30, 60, 90, 120, 150, 180, 210, and 240 min relative to injection to assess the pituitary-gonadal response to GnRH. These blood samples were analyzed for concentrations of LH, FSH, and testosterone.

Semen collection was conducted daily for 6 d starting 30 d after plasmid injection. Semen evaluation (Pickett et al., 1976) was conducted on the last 3 ejaculates from each stallion. Gel volume, gel-free volume, progressive motility, concentration, and general sperm morphology were evaluated. Morphological characteristics (head, midpiece, and tail abnormalities and proximal and distal droplets) were assessed in gel-free semen fixed in 2% buffered formol-saline; 100 sperm from each ejaculate were analyzed by viewing under phase contrast microscopy (Blanchard et al., 2003).

Blood collected during frequent and daily sampling was immediately centrifuged (1,600 x g at 5°C for 15 min) and plasma was harvested and stored at -15°C until assay. All samples were analyzed as previously described for SEAP (Phospha-Light System), LH (Thompson et al., 1983a), FSH (Thompson et al., 1983b), and testosterone (Diagnostic Systems Laboratories, Webster, TX).

Tissue culture data were analyzed using t-test to compare the mean of the 3 transfected cell cultures to zero; there was only one nontransfected culture, and there was no detectable GnRH in that medium.

Data obtained from blood collections in the stallion experiment were analyzed for effects of treatment and time and the treatment by time interaction as a randomized block design with repeated measures using SAS mixed procedure (SAS Institute Inc., Cary, NC). Seminal characteristics were analyzed for effects of treatment and time and the treatment by time interaction via the mixed procedures of SAS. Differences at individual time points were

determined by LSD test when a significant F ($P < 0.05$) was detected. Plasma concentrations of LH, FSH, testosterone and SEAP were adjusted to net differences from pretreatment means prior to analysis to account for individual variation in resting hormone concentrations.

Results

Tissue culture of Sol8 mouse myoblast cells transfected with pGnRH or no DNA indicated increased ($P < 0.01$) expression in cells transfected with pGnRH (Figure 3.1). In the stallions, responses for the 2 mg and 4 mg DNA injection were similar in treated stallions; thus, the groups were combined and referred to collectively as "pGnRH-treated." Treatment with pGnRH increased ($P < 0.05$) plasma concentrations of testosterone above controls in blood samples collected twice weekly by d 35 post treatment (Figure 3.2). The increase in testosterone concentrations from treated stallions was maintained for the duration of sample collection (d 58). Plasma concentrations of LH and FSH in samples collected twice weekly were not different ($P > 0.1$) between treatment groups (Figure 3.2). Frequent blood sampling conducted around the GnRH challenge revealed an increased ($P < 0.01$) response in plasma LH concentrations and a tendency ($P = 0.098$) for increased response in testosterone concentrations (Figure 3.3). Conversely, concentrations of FSH were similar ($P > 0.1$) between groups in response to the GnRH challenge (Figure 3.3). Control stallions responded to pSEAP with increased ($P < 0.01$) plasma concentrations of SEAP (Figure 3.4). Seminal characteristics did not differ ($P > 0.1$) between groups (Figure 3.5).

Discussion

Treatment with pGnRH increased testosterone production gradually through the end of the sampling period (58 d). Similarly, Blue and colleagues (1991) reported an increase in testosterone secretion in stallions in response to long-term, pulsatile (10 μg every 2 h)

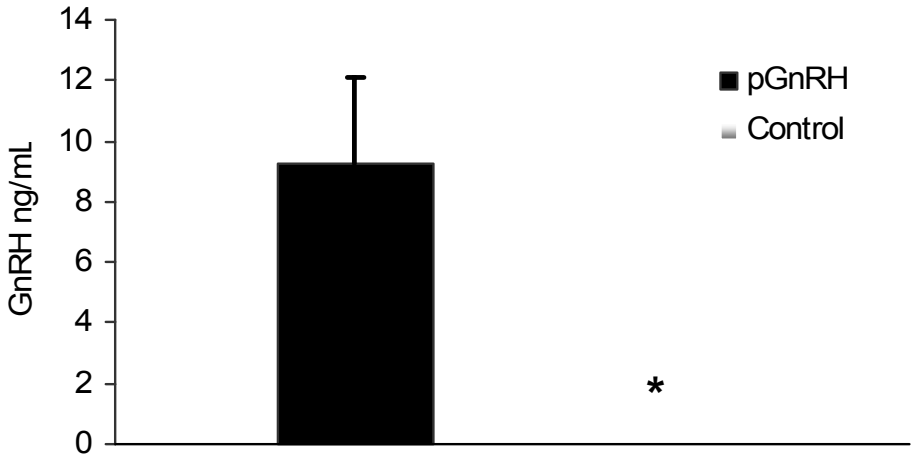

Figure 3.1. Tissue culture concentrations of GnRH in media harvested from Sol8 mouse myoblasts cells transfected with pGnRH or no DNA (control). Cells transfected with pGnRH had increased ($P < 0.01$) expression of GnRH. The asterisk indicates GnRH concentration was below detectable levels. The vertical bar indicates 2 standard deviations from the mean (95% confidence interval).

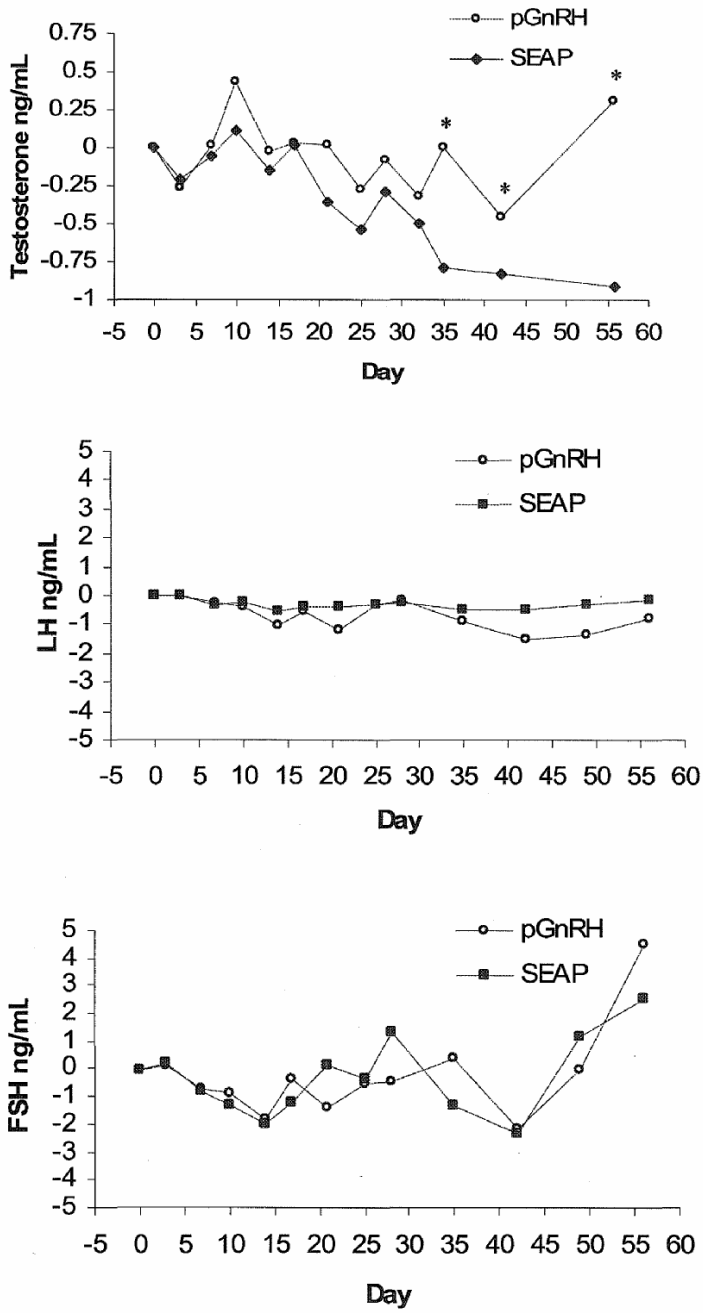

Figure 3.2. Mean plasma concentrations expressed as net differences from pretreatment means for testosterone, LH, and FSH in samples collected twice weekly after pGnRH and pSEAP treatment on d 0. Testosterone concentrations increased ($P < 0.05$) in pGnRH-treated stallions. Concentrations of LH and FSH were similar between groups. Asterisks indicate differences between groups ($P < 0.05$). Pooled SEM were 0.33, 0.46, and 0.81 for testosterone, LH, and FSH, respectively.

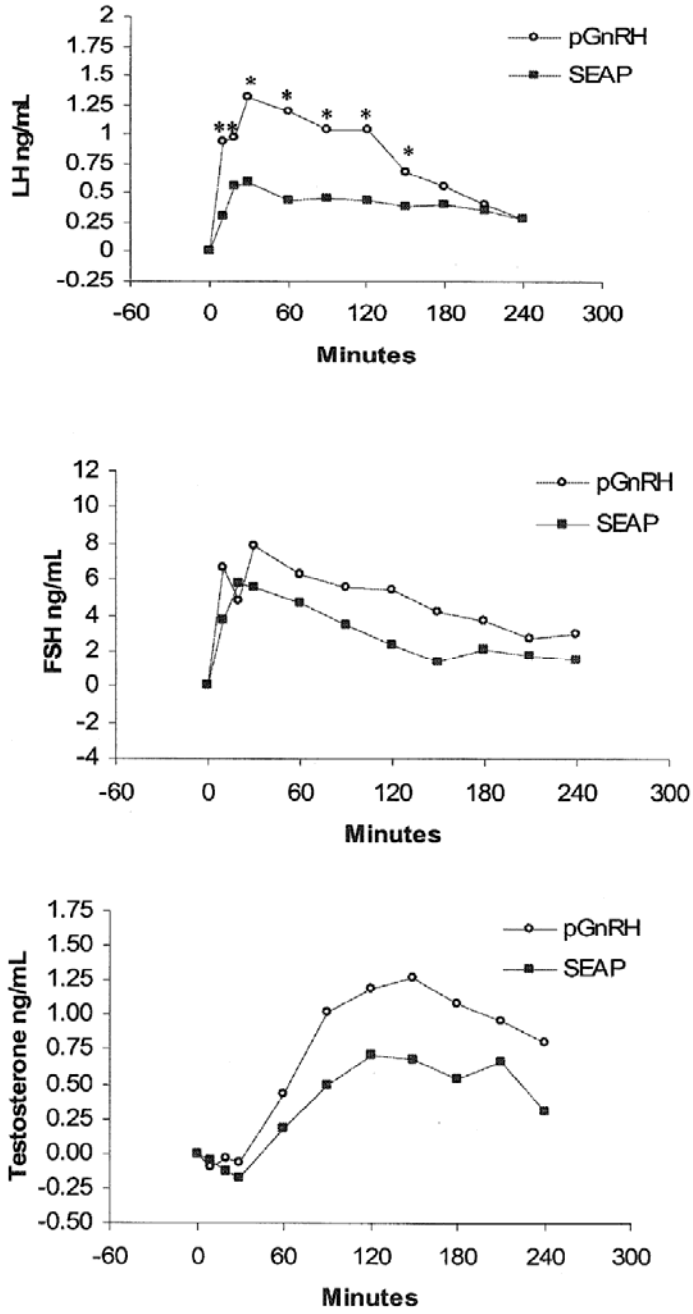

Figure 3.3. Plasma concentrations expressed as net differences from pretreatment means for testosterone in pGnRH- and pSEAP-treated stallions after GnRH administration (time 0) on d 21. Stallions treated with pGnRH had a greater ($P < 0.05$) LH response to GnRH; concentrations of FSH were similar between groups. Treatment with pGnRH tended to increase ($P < 0.1$) the testosterone response to GnRH administration. Asterisks indicate differences between means ($P < 0.05$). Pooled SEM were 0.22, 3.8, and 0.24 ng/mL for LH, FSH, and testosterone, respectively.

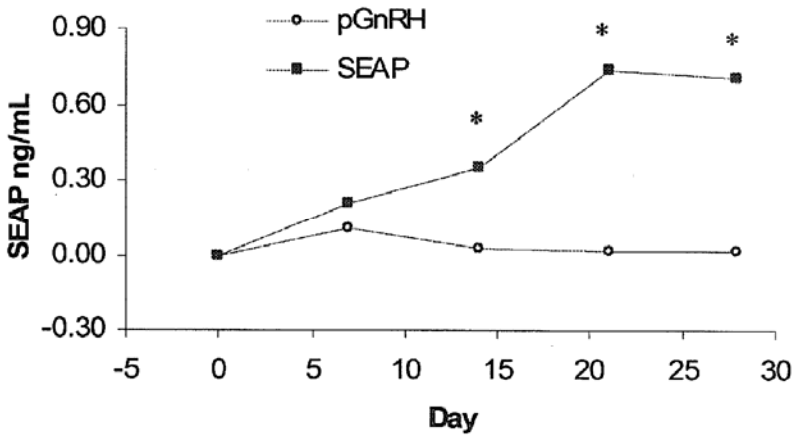

Figure 3.4. Plasma concentrations of SEAP in pGnRH- and pSEAP-treated stallions expressed as net difference from pretreatment means. Treatment with pSEAP increased ($P < 0.05$) plasma concentrations of SEAP. Asterisks indicate differences between groups ($P < 0.05$). Pooled SEM was 0.15 ng/mL.

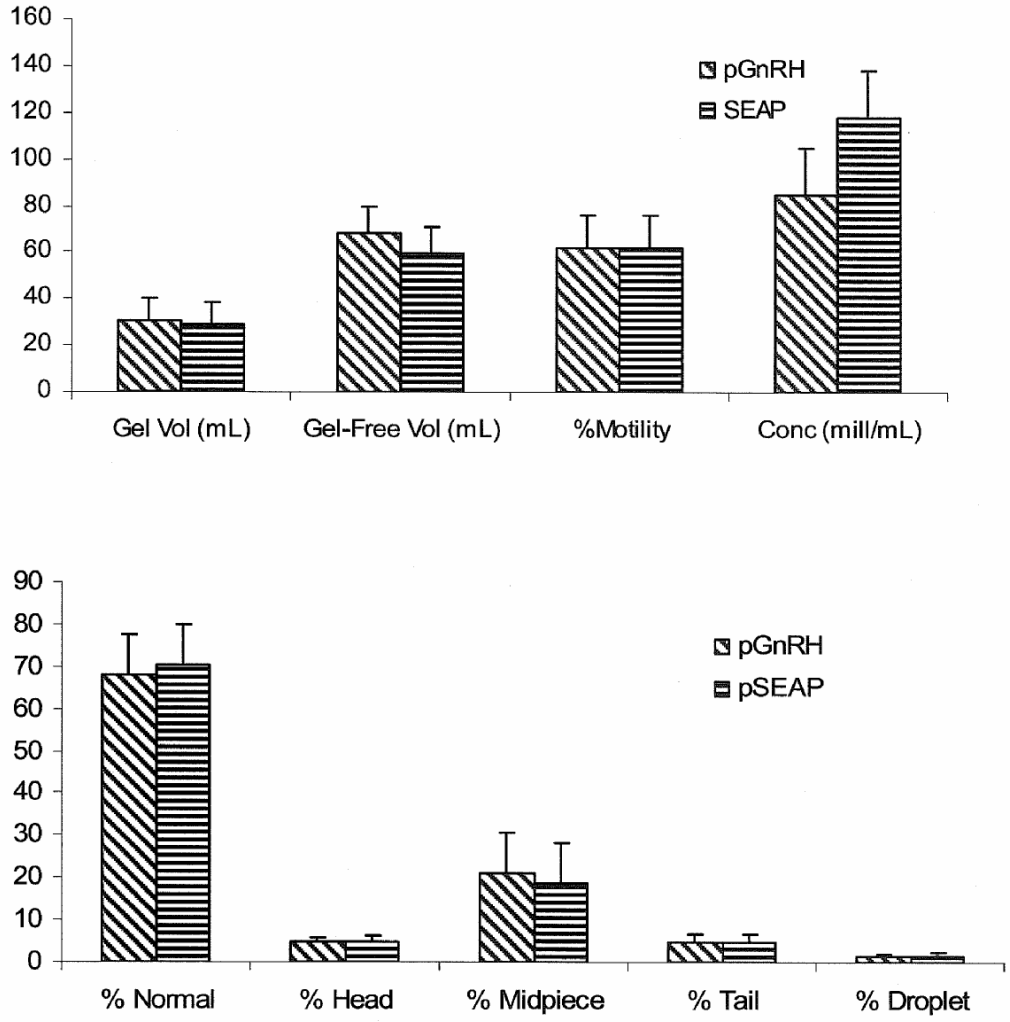

Figure 3.5. Seminal characteristics from stallions treated with pGnRH or pSEAP and evaluated on d 30 relative to treatment. Seminal characteristics evaluated were similar ($P > 0.1$) between groups. Error bars indicate pooled SEM for each characteristic.

administration of GnRH. Blue et al. (1991) also reported that continuous infusion of GnRH at a similar rate was not effective at increasing testosterone or LH secretion. The exact nature of GnRH expression from pGnRH in this experiment is unknown. Because plasma concentrations of GnRH were not directly measured, it is assumed that expression is similar to that determined for SEAP. However, compared to GnRH, SEAP has a relatively long half-life in peripheral blood, therefore its concentrations may not necessarily be indicative of the secretion of the short half-life peptide. Based on this information, pGnRH expression may be more physiological than constant infusion of GnRH, thereby enabling an effect on testosterone secretion as is seen with pulsatile administration.

Treatment with pGnRH was not effective at increasing basal concentrations of gonadotropins but did increase the LH response to a physiologic dose of GnRH on d 21. It is likely that increasing concentrations of testosterone provided negative feed back adequate to inhibit LH secretion from the pituitary (Thompson et al., 1979) while production was still being stimulated by GnRH. In anestrous mares, daily injections of a GnRH analogue potentiated the LH response to each consecutive injection, while basal LH concentrations between GnRH injections were similar to controls (Gentry et al., 2002). This indicates an ability of GnRH to increase intracellular production of LH without noticeable increases in basal secretion. In the present experiment, intracellular production of LH may have been augmented at the same time that steroid inhibition was minimizing LH secretion. Intuitively, increased levels of testosterone should have been accompanied by a reduction in LH secretion. Maintenance of basal LH concentrations in the treated group, therefore, likely indicated a positive effect of pGnRH.

Horses have been reported to be fairly resistant to down-regulation by GnRH and its analogs. Boyle and colleagues (1991) used osmotic pumps to deliver buserelin (a potent GnRH analogue) to assess the long-term effects of high levels of GnRH. In their study, LH and

testosterone concentrations were initially increased and subsequently decreased below baseline. These data are in contrast to the results herein, indicating GnRH expression from pGnRH did not attain levels required for down-regulation. Current interest in the horse industry has included utilization of potent analogs for regulating reproductive related behavior. Future efforts might be directed towards evaluating the effects of improved expression or incorporating genetic expression of GnRH analogs into the plasmid for treatment of reproductive related behavior in the horse.

Seminal characteristics were not altered when semen was evaluated on d 34 through 36, in spite of the increased testosterone secretion. Blue and colleagues (1991) similarly reported that constant infusion or pulsatile administration of GnRH for 5 mo did not alter total number of spermatozoa or progressive motility. This may reflect the lack of perturbation in basal LH and FSH secretion in response to pGnRH treatment. Also, considerable differences in testosterone secretion were not evident until d 35. Differences attributed to the variation in testosterone secretion may have been more evident if semen collection had been conducted later in the study.

Increased concentrations of SEAP in pSEAP-treated stallions confirmed the functionality of the technology in the stallion. As stated previously, SEAP concentrations may not adequately reflect the expression pattern of GnRH. The utilization of pSEAP as a control demonstrated that the electroporation technique and the plasmid backbone operated appropriately. Considering that the pGnRH contains a similar backbone and was delivered with the same technique, it is assumed that GnRH was expressed similar to SEAP, except that GnRH should not elicit an immune response due to its small size and natural occurrence in the horse.

In conclusion, intramuscular plasmid delivery followed by electroporation seems to have potential as an effective hormone delivery system for the horse. The current experiment indicated that the basic components of the GHRH and SEAP plasmids can be used to construct

plasmids containing the DNA of other potentially beneficial peptides. Such a plasmid was used to facilitate evaluation of the effects of GnRH on the reproductive axis of the stallion. Increased testosterone production in the stallion was accompanied by a perturbation in LH secretion after GnRH challenge. Furthermore, the increase in testosterone production was not associated with adverse physiological effects with regard to down-regulation of gonadotropins or semen morphology. These results warrant future research into the possible beneficial uses of this technology for the equine industry.

CHAPTER IV

REGULATION OF THE REPRODUCTIVE AXIS OF GOAT DOES BY ELECTROPORATIC DELIVERY OF A PLASMID ENCODING GNRH

Introduction

Reproductive seasonality is a natural phenomenon of many domesticated and exotic species. The seasonal variation in reproductive capacity for each species is relative to their gestation length, allowing for birth of offspring during the most fertile environmental period. Environmental factors, including photoperiod, temperature, and nutrition, are influential factors regulating seasonal reproductive recrudescence (Sharp, 1988; Gentry et al., 2002). These environmental stimuli indirectly regulate reproduction by modulating hypothalamic GnRH secretion. Treatment with GnRH and its analogs have shown promise at inducing reproductive recrudescence during deep anestrus (Johnson, 1987; Turner and Irvine, 1991; Medan et al., 2002). Problems have arisen with these treatments due to an inability to mimic physiologic GnRH secretion. Native GnRH is short-lived in circulation and requires frequent administration or continuous infusion to mimic physiologic secretion. Potent analogs tend to down-regulate gonadotropin production, especially in ruminants. A more physiologic and long-term GnRH therapy is needed to further investigate the potential use of GnRH in goat production systems. The goal of the current experiment was to develop a plasmid-based peptide delivery system for the goat and to utilize the delivery system to produce a long-term GnRH therapy for the anestrous doe in an attempt to reverse the seasonal anestrous state.

Materials and Methods

Twenty anestrous doe goats ranging in age from 2 to 6 yr were selected on June 19, 2006. Does were maintained on a pelleted ration and supplemental grass hay as needed to maintain body condition. A doe was determined to be seasonally anestrus based on plasma progesterone

concentrations less than 0.5 ng/mL for 10 d with no signs of estrous behavior. Does were paired based on age and weight and were randomly allotted from each pair into 2 treatment groups. The groups received i.m. electroporatic delivery of the plasmid pGnRH or pSEAP (1 mg DNA in 1 mL WFI + 0.1% PLG; n = 10 per group), respectively. The plasmids were injected into the semimembranosus muscle as described in Chapter II.

On d 21, 5 does from each treatment group were each implanted with one-half of a Crestar implant (3 mg norgestomet; Intervet International Boxmeer, The Netherlands), which was left in place for 10 d. Blood samples were collected from all does via jugular venipuncture into heparinized tubes every 3 to 4 d for 28 d with 2 pretreatment blood samples collected on d -3 and d 0 relative to treatment. Additional blood samples were collected on d 29 to 58 from implanted does to characterize the response in plasma gonadotropins to implantation. Does were exposed to a sexually vigorous buck twice daily beginning 2 wk before treatment for the detection of estrus.

All blood samples were immediately centrifuged (1,600 x g at 5°C for 15 min) after collection, and plasma was harvested and stored at -15°C. All samples were analyzed as previously described for SEAP (Phospha-Light System), LH (Thompson et al., 1983a), FSH (Thompson et al., 1983b), and progesterone (Diagnostic Systems Laboratories, Webster, TX).

Data from blood samples were analyzed for effects of treatment and time and the treatment by time interaction as a randomized block design with repeated measures using SAS mixed procedure (SAS Institute Inc., Cary, NC). Data were analyzed through d 28 on all does, and an additional analysis was conducted separately for d 21 to 58 on implanted does. Differences at individual time points were determined by LSD test when a significant F ($P < 0.05$) was detected. To account for individual variation in resting hormone concentrations prior to treatment, plasma concentrations of LH, FSH, and SEAP were calculated as the net

differences from pretreatment means prior to analysis. Pretreatment means of each individual were subtracted from their subsequent data, and the analyses were performed on the differences.

Results

Treatment with pGnRH did not influence ($P > 0.1$) plasma concentrations of LH and FSH collected over the first 28 d (Figure 4.1). As expected, does treated with pSEAP had increased ($P < 0.01$) plasma concentrations of SEAP that returned to baseline by d 28 after treatment (Figure 4.1). Plasma concentrations of LH and FSH in blood samples collected from implanted does gradually increased over time ($P < 0.05$) but were similar ($P > 0.1$) for the 2 treatment groups (Figure 4.2). Implantation on d 21 did not disrupt ($P > 0.1$) basal concentrations of gonadotropins or induce estrus in either treatment group. Lack of ovulation was indicated by low levels of progesterone throughout the blood sampling period for both groups. Estrus detection, performed by twice daily exposure to the buck, revealed minimal signs of sporadic estrus approximately 20 d after exposure to the buck in both treatment groups; these were not associated with mounting or changes in measured hormonal parameters.

Discussion

The primary goal of this experiment was to develop a mammalian plasmid-based gene transfer system for the goat. Treatment with pSEAP increased plasma concentrations of SEAP in the control group as expected. This confirmed that the pSEAP plasmid was effectively delivered into the muscle of the goats, and SEAP was expressed and secreted. Concentrations of SEAP peaked around d 15 after treatment, similar to SEAP expression produced in previous experiments conducted in horses. Duration of SEAP expression, as indicated by plasma concentrations, is believed to be limited by the immunogenicity of SEAP in adult mammals, whereas, expression of immunoneutral protein products would be expected to persist beyond the

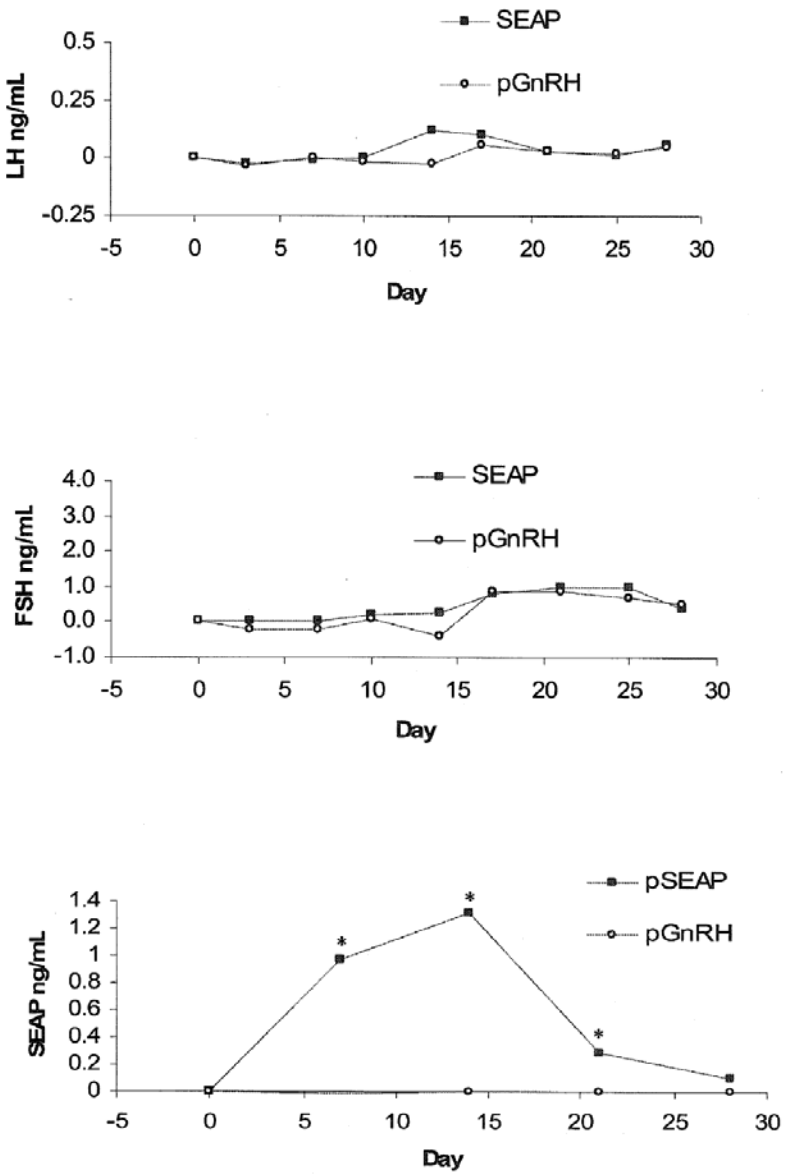

Figure 4.1. Mean plasma concentrations expressed as net differences from pretreatment means for LH, FSH, and SEAP in blood samples collected twice weekly from does relative to treatment with pGnRH or pSEAP on d 0. Concentrations of LH and FSH were similar between treatment groups. Concentrations of SEAP were increased ($P < 0.05$) in response to pSEAP treatment. Asterisks indicate differences between means ($P < 0.05$). Pooled SEM were 0.05, 0.33, and 0.13 ng/mL for LH, FSH, and SEAP, respectively.

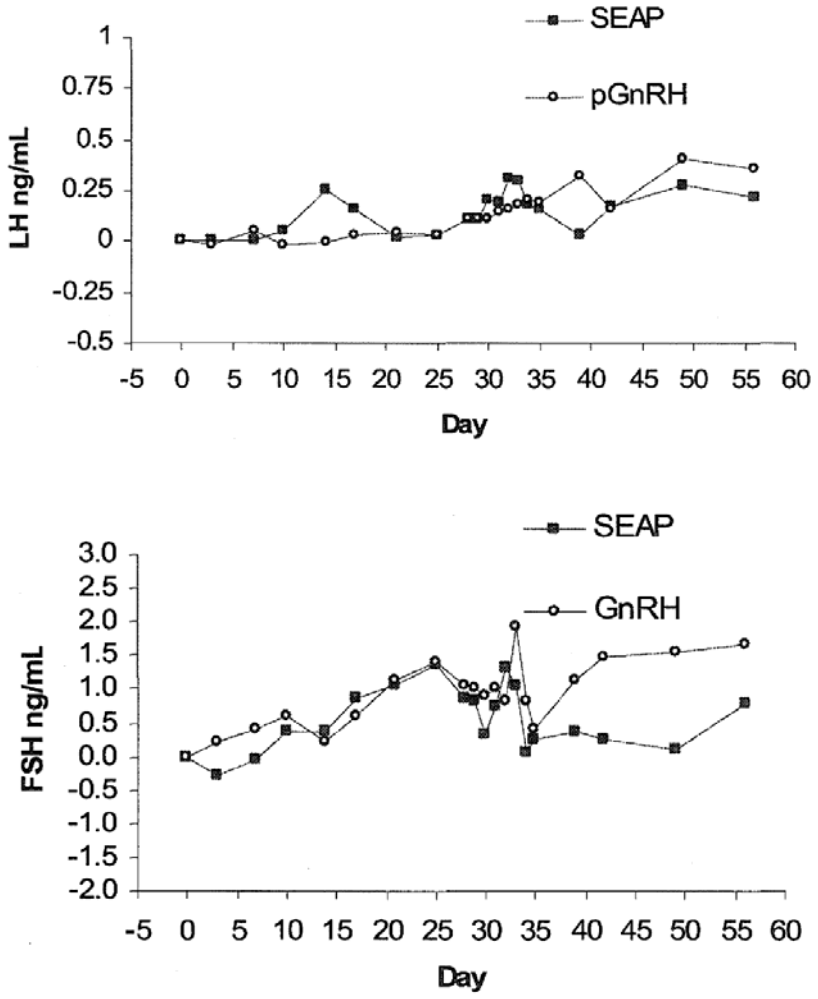

Figure 4.2. Mean plasma concentrations expressed as net differences from pretreatment means for LH and FSH in does treated with pGnRH or pSEAP on d 0 and implanted with progesterone (d 21 to 31). Concentrations of LH and FSH increased over time ($P < 0.05$) in both treatment groups. No differences were attributed to pGnRH treatment. Pooled SEM were 0.11 and 0.84 ng/mL for LH and FSH, respectively.

30 d in the pSEAP control. If the pGnRH plasmid were delivered and expressed as effectively as the pSEAP plasmid, expression of pGnRH should have been adequate to elicit a desirable outcome (increased gonadotropin secretion and ovarian activity).

A secondary goal of this experiment was to establish a GnRH secreting plasmid and determine the effects of long-term GnRH therapy on the anestrous doe. Given the results herein, the efficacy of the plasmid for expression in the doe cannot be determined. In stallions (Chapter III), treatment with pGnRH resulted in a gradual increase in testosterone throughout the blood sampling period (56 d) indicating a positive effect of the plasmid in the horse. It is likely that does treated with pGnRH responded with expression of GnRH but the levels produced were not sufficient to increase gonadotropins in the anestrous state. Further research is needed to determine whether does exhibiting nadir levels of gonadotropins during anestrus are capable of responding to the pGnRH treatment.

Current schemes for inducing ovulation in anestrous does include progesterone pessaries in conjunction with GnRH or gonadotropins (Bretzlaff and Romano, 2001; Whitley and Jackson, 2004). Progesterone-priming in conjunction with hourly injections of GnRH was capable of inducing an LH surge and ovulation in seasonally anoestrous goats (Knight et al., 1988). Therefore, the progesterone implant administered on d 21 after plasmid delivery, if accompanied by sufficient GnRH secretion, should have been able to initiate ovulation. Conversely, continuous infusion of GnRH has been shown to result in down-regulation of gonadotropin secretion (Porter et al., 1997b). Although it is possible that expression of GnRH from pGnRH overwhelmed the pituitary resulting in a down-regulation of receptors on the gonadotropes, it is probably more likely that insufficient GnRH was secreted to cause an effect.

In conclusion, electroporation of plasmid DNA encoding SEAP did function in the goat in a manner and to a degree similar to that observed other species, indicating that this technology

may have potential for applications in goat management schemes and veterinary therapy. The role of GnRH in the anestrous doe could not be determined. The anestrous doe may not be responsive to chronic, low-level GnRH treatment. Future efforts are needed to further evaluate the applications of this novel technology in domestic livestock.

SUMMARY AND CONCLUSIONS

A novel, plasmid-based peptide delivery technique was studied in the horse and the goat. The technique was effective in producing peptide products in vivo in both species. Treatment with pSEAP resulted in increased plasma concentrations of SEAP in all instances. Efforts at exploiting the technique for increasing GHRH and GnRH in the stallion were successful within limits, as determined by elevated plasma concentrations of IGF-I and testosterone, respectively. Treatment with pGHRH may have improved the seminal quality by increasing the number of normal spermatozoa and decreasing the number of sperm head abnormalities. This treatment may have potential in the horse industry as a treatment for infertility in popular or geriatric breeding stallions. Elevated levels of testosterone resulting from treatment with pGnRH could facilitate improved performance and libido in racing and breeding stallions.

Trials aimed at increasing GnRH in the anestrous doe were inconclusive. It could not be determined whether pGnRH was not expressed in the does, or whether the expression and secretion were too low to have effects on LH and FSH secretion. It is also possible that a chronic, low-level GnRH secretion down-regulated gonadotropins in the doe rather than stimulating them. A more pulsatile secretion pattern might be necessary to elicit LH secretion accompanied by ovulation.

Although these first attempts to apply this novel plasmid delivery system to horses and goats produced limited positive effects, this area of research has potentially limitless applications for domestic livestock production and human medicine. Subsequent research needs to focus on the fine tuning of the technology to maximize the expression and secretion of produced peptides.

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VITA

William Andrew Storer, son of George and Courtney Storer, was born in Moss Bluff, Louisiana, on April 4, 1976. William is the oldest of four children. He resided in Moss Bluff until the age of 18 and attended high school at Sam Houston High. After graduating in 1994, William was accepted into McNeese State Univeristy in Lake Charles, Louisiana, where he earned a bachelor of science degree in agriculture. In August of 2000, William began working towards a master of science degree in animal sciences at Louisiana State University; he married Kristina Hope Barnett the following year. In August of 2002, he began the pursuit of the doctoral degree in animal sciences at Louisiana State University, and became the proud father of Cameron William Storer in 2005. After completing his doctorate, William plans to move to Lake Charles, Louisiana, to begin teaching at McNeese State University.